PRODUCTION OF BIOETHANOL FROM TAPIOCA STARCH USING *Saccharomyces cerevisiae*: EFFECTS OF TEMPERATURE AND AGITATION SPEED

MUHAMAD FAUZI BIN IBRAHIM

A thesis submitted in fulfillment
of the requirements for the award of the degree of
Bachelor of Chemical Engineering (Biotechnology)

Faculty of Chemical & Natural Resources Engineering
Universiti Malaysia Pahang

April 2009

ABSTRACT

Production of bioethanol from tapioca starch involves two processes which are enzymatic hydrolysis and fermentation. The objective of the present study was to investigate the influences of temperature and agitation speed on the production of bioethanol from tapioca starch using *Saccharomyces cerevisiae.* The fermentation was conducted under various temperatures (30, 35 and 37°C) and agitation speeds (100, 200 and 300 rpm) in 250 mL shake flask. The cell density, glucose consumption and ethanol concentration were analyzed. The ethanol concentration in the fermentation broth increased rapidly with the increased of temperature and agitation speed. The high temperature resulted in the higher cell density and higher glucose consumption. The high agitation speeds also preferred for both cell density and glucose consumption. The maximum ethanol concentration of 57.8 mg/L was obtained at a temperature of 35°C and 200 rpm of agitation speed.

ABSTRAK

Penghasilan bioetanol daripada kanji ubi kayu melibatkan dua proses iaitu hidrolisis dan penampaian. Objektif bagi kajian ini adalah untuk mengenalpasti pengaruh suhu dan halaju adukan dalam penghasilan bioetanol daripada kanji ubi kayu mengunakan *Saccharomyces cerevisiae*. Proses penapaian dijalankan pada pelbagai keadaan suhu (30, 35 and 37°C) dan halaju adukan (100, 200 and 300 rpm) di dalam kelalang 250 mL. Kepadatan sel, pengunaan glukosa dan kepekatan etanol di analisa. Kepekatan etanol meningkat dengan ketara dengan peningkatan suhu dan halaju adukan. Kenaikan suhu penampaian menyebabkan peningkatan kepadatan sel dan peningkatan penggunaan glukosa. Halaju adukan yang tinggi pula adalah lebih sesuai untuk penghasilan kepadatan sel dan penggunaan glukosa. Kepekatan etanol paling maksimum adalah 57.8 mg/L diperolehi pada suhu 35°C dan halaju adukan 200 rpm.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS/ABBREVIATIONS

C	-	carbon
CO_2	-	carbon dioxide
DNS	-	Di-Nitro Salicylic Acid
g	-	gram
g/L	-	gram per liter
H	-	hydrogen
hr	-	hour
Mg^{2+}	-	ion magnesium
min	-	minutes
mL	-	milliliter
Mg	-	magnesium
mg	-	milligram
mmol	-	milimole
Na	-	sodium
OD	-	optical density
rpm	-	rotation per minute
w/v	-	weight per volume
%	-	percentage
°C	-	degree celsius
μL	-	microliter

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Ethanol is known as ethyl alcohol or fermentation alcohol. It is referred to one type of alcohols found in alcoholic beverages (Wyman, 2004). Due to the unstable price and the availability of crude petroleum, the fermentation has become an alternatives process to produce ethanol (Logsdon, 1994). Baras *et al*., (2002) had reported that almost 60% of the ethanol is produced by fermentation where the major world producers are Brazil and the US, which together account for about 80% of the world production. Mostly ethanol produced had been widely used in cars as a fuel alternative to gasoline.

Bioethanol is easy to manufacture and process since the raw materials used as the feedstock is unlimited and cheaper. Major carbohydrate-containing substrates such as cane, beet, sago and corn are used for a feedstock in ethanol production and commonly available in tropical countries (Ramasamy *et al*., 2001). Starchy substrate such as tapioca could be exploited for ethanol production. The content of tapioca composed of 95% starch and 2% moisture. Owing to its high carbohydrate content, tapioca becomes one of the most efficient sources of starch. This raw material has not yet been fully exploited in highly technical industries for ethanol production. Since the use of starch-based raw materials for ethanol production is not a common practice, it is important to determine the optimized conditions for starch processing in order to enhance the bioethanol utilization in Malaysia (Aggarwal *et al*., 2001).

The hydrolysis of starch may be considered as a key step in substrate processing for bioethanol production. The main role of this step is to effectively provide the conversion of two major starch polymer components of amylase and amylopectin. Another crucial step would be the fermentation process that could subsequently be converted to ethanol by yeasts or bacteria (Mojovic *et al*., 2006). The parameters involve mainly pH, temperature, agitation speed, inoculum age, medium etc. have to be evaluated and optimized in order to obtain a good yield of bioethanol.

1.2 Objectives

The aim of this study is to determine the optimum conditions of fermentation process for the production of bioethanol from tapioca starch. The objectives of this research are:

- To determine the effect of temperature on the production of bioethanol from tapioca starch using ***Saccharomyces cerevisiae.***
- To determine the effect of agitation speed on the production of bioethanol from tapioca starch using ***Saccharomyces cerevisiae***.

1.3 Scope of the Study

Bioethanol production process has two steps which are enzymatic hydrolysis and fermentation process. The scope for this study was to determine the yield of bioethanol that can be produced using tapioca starch in fermentation process. The 250 mL fermentation will conducted to investigate the effects of temperature and agitation speed in fermentation process. The optimum bioethanol production from tapioca starch using *Saccharomyces cerevisiae* was aimed. Others parameters such as pH, dissolved oxygen, nutrient and time were fixed during the process study.

1.4 Problem Statement

A long time ago until now, gasoline u.sages have a higher demand every year. In recently, the world face a crisis of diminishing fossil fuel reserves, thus an alternative energy sources need to be renewable, sustainable, efficient, cost-effective, convenient and safe (Chum and Overend, 2001).

In June 2008, the price of Malaysia gasoline increased by 40% from RM1.92/litre to RM2.70/litre. In order to reduce the use a large amount the gasoline in daily, Malaysian researches and development have focused on commercially produced ethanol as an alternative fuel. Fermentative production of ethanol from renewable resources has received attention due to increasing petroleum shortage. Most of the raw materials utilized for bioethanol production were corn grain and sugar cane (Mojovic *et al*., 2006). It is important to see the potential of raw materials rich in fermentable carbohydrates such as tapioca since it is largely available in Malaysia.

Gasoline is a finite resource that cannot be sustained indefinitely, and its price is increased as its availability is decreased over time. Gasoline can produce toxic substances and gaseous emissions when it is combusted. It will cause the negative impact on the environment, particularly greenhouse gas emissions and these problems have warned the society to find another renewable fuel as an alternative. Bioethanol on the other hand is most environmental friendly. It is known as a high octane fuel with lower emissions can be use in car as fuel. It can be produced using cheaper materials biologically for feedstock and is already compatible, in low blends, with existing gas engines (Wyman, 2004).

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of Ethanol

In recent years, ethanol is one of the most important renewable fuels contributing to the reduction of negative environmental impacts generated by the worldwide utilization of fossil fuels (Cardona and Sanchez, 2007). Due to the diminishing fossil fuel reserve, research and development efforts directed toward commercial production of ethanol from renewable resources have increased (Mojovic *et al*., 2006).

Brazil and the United States lead the industrial world in global ethanol production, accounting together for 70% of the world's production and nearly 90% of ethanol is used for fuel. In 2006, Brazil has produced 16.3 billion liters of ethanol represents 33.3% of the world's total ethanol production. Sugar cane plantations cover 3.6 million hectares of land for ethanol production with a productivity of 7,500 liters of ethanol per hectare. The U.S. in the other hand, 3,000 liters per hectare of maize ethanol was produced (Cardona and Sanchez, 2007).

Fermentation alcohol has been investigated for several years. Substrate used for this process essentially depends on surplus grains production of each country. In the United States maize was used whereas, in France, the principal cereal for alcohol production is wheat. Several studies on alcohol have been carried from cellulosic biomass, cassava, sago, sorghum, blackstrap molasses, and maize but few on raw wheat flour as substrate. Figure 2.1 shows the countries that have the worlds least cost of ethanol production.

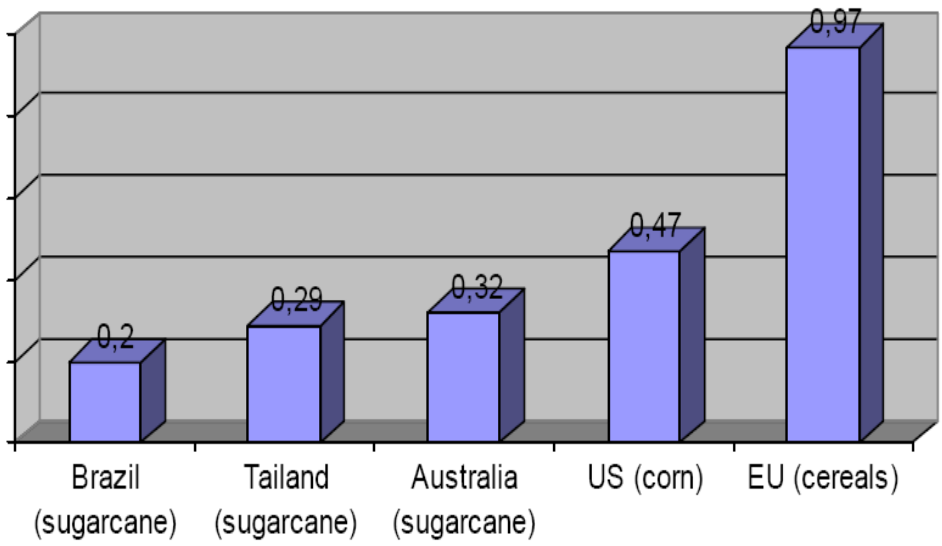

Figure 2.1: Ethanol production costs (US$ per liter) (Salomao, 2005)

In daily application, ethanol is mostly used as fuels (92%), industrial solvents and chemicals (4%) and beverages (4%) (Logsdon, 2006). An important issue regarding the ethanol production is weather the process is economical. Research efforts are focused to design and improve a process, which would produce a sustainable transportation fuel. A low cost of feedstock is a very important factor in establishing a cost effective technology (Mojovic *et al*., 2006). Therefore, a strong need exists for efficient ethanol production with low cost raw material and production process (Liu, 2007).

2.2 Ethanol Use as a Fuel

Ethanol is known as ethyl alcohol or fermentation alcohol, often referred to as just ‘‘alcohol,’ and has the chemical formula of C_2H_5OH. It is a colorless, clear liquid that looks like water and is completely miscible with water. Ethanol has a somewhat sweet flavor when diluted with water; a more pungent, burning taste when concentrated; and an agreeable ether-like odor. Other that, it is more volatile than water, flammable, burns with a light blue flame, and has excellent fuel properties for spark ignition internal combustion engines (Wyman, 2004).

Ethanol has decreased the need for other octane booster such as benzene, which are toxic and often carcinogenic. The oxygen in ethanol reduces emissions of burned hydrocarbons and carbon monoxide, particularly for older vehicles that tend to burn rich (Wyman, 2004). As an oxygenated compound, ethanol provides additional oxygen in combustion, and hence obtains better combustion efficiency. Since the completeness of combustion is increased by the present of oxygenated fuels, the emission of carbon monoxide is reduced by 32.5% while the emission of hydrocarbon is decreased by 14.5% (Rasskazchikova *et al*., 2004). Nguyen *et al*., (1996) has stated the ethanol has lower environmental impact than petrol in terms of the combustion by-products and greenhouse gases.

The use of ethanol as fuel goes back to origin of the use of vehicles itself. For example, Henry Ford’s Model T. built in 1908, ran on ethanol. It was continued until the availability of cheap petrol effectively killed off ethanol as a major transport fuel in the early part of the 20th century. The energy crisis of the 1970s then renewed interest in ethanol production for fuels and chemicals (Marrs, 1975).

2.3 Economics and Environmental Impact of Ethanol Production

Ethanol made from biomass provides unique environmental, economic strategic benefits and can be considered as a safe and cleanest liquid fuel alternative to fossil fuels. The ethanol yields and process economics along with the technical maturity and environmental benefits are the key parameters that determine the feasibility of bioethanol production (Nguyen and Saddler, 1991). The use of bioethanol as a source of energy would be more than just complementing for solar, wind and other intermittent renewable energy sources in the long run (Lin and Tanaka., 2006).

Ethanol has provided multiple economic, social and environmental benefits to the producing regions and to the country as a whole. Ethanol has made a significant contribution to the reduction of greenhouse gas emissions, substituting ethanol for gasoline for fossil fuels (Lucon *et al*., 2005). Furthermore, ethanol represents closed carbon dioxide cycle because after burning of ethanol, the released carbon dioxide is recycled back into plant material because plants use CO_2 to synthesize cellulose during photosynthesis cycle (Wyman, 2004). Ethanol production process only uses energy from renewable energy sources. Hence, no net carbon dioxide is added to the atmosphere, making ethanol an environmentally beneficial energy source (Figure 2.2).

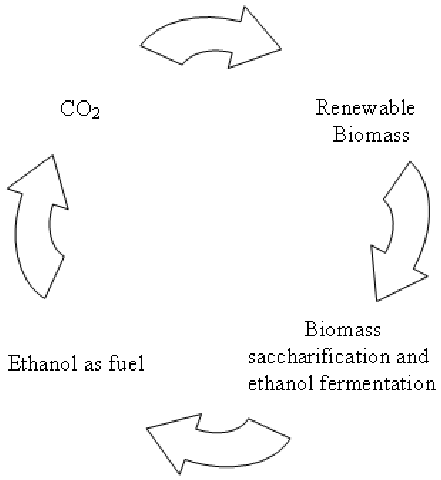

Figure 2.2 Ethanol represents closed CO_2 cycle (Chandel *et al*., 2007).

2.4 Enzymes

2.4.1 α-Amylases.

α-Amylasesare obtained from *Aspergillus oryzae*, *Bacillus amyloliquefaciens*, and *Bacillus licheniformis* are mostly used in starch hydrolysis for sugar syrups and brewing. The end products from the action of a-amylase on starch are glucose, maltose, maltotriose. maltotetraose. maltopentaose, and maltohexaose (Nigam and Singh., 1994)

2.4.2 Glucoamylases.

Glucoamylases obtained *Aspergillus niger* and *Rhizopus* species are used in glucose syrup production from liquefied starch (Nigam and Singh., 1994).

2.5 *Saccharomyces cerevisiae*

Saccharomyces cerevisiae (brewer's and baker's yeast) has been used in classical food fermentation, the production of fuel alcohol, glycerol, invertase and animal feeding (Camacho-Ruiz *et al*., 2003). *Saccharomyces cerevisiae* is found in the wild growing on the skins of grapes and other fruits. There are two ways *Saccharomyces cerevisiae* breaks down glucose which are aerobic and anaerobic conditions**.** *Saccharomyces cerevisiae* strains for ethanol production due to its high ethanol yield and high tolerance to rather high ethanol concentration.

2.6 Starch

Starch, chemical formula $(C_6H_{10}O_5)_n$ is a polysaccharide carbohydrate consisting of a large number of glucose units joined together by glycosidic bonds. It is consisting of amylase (linear chain of glucose) and amylopectic (branched chain of glucose). Starches are found in a large number of plants as the major carbohydrates reserve and provide an essential source of energy. The common source of starch comes from maize, wheat, potatoes, tapioca and rice (Marrs, 1975).

Starch of tapioca is a major starch sources and has a wide commercial use. A typical composition of the tapioca root is moisture (70%), starch (24%), fiber (2%), protein (1%) and other substances including minerals (3%). Tapioca ranks very high among crops that convert the greatest amount of solar energy into soluble carbohydrates per unit of area. Among the starchy staples, tapioca gives a carbohydrate production which is about 40% higher than rice and 25 % more than maize. Besides, tapioca is cheapest sources of calories for both human nutrition and animal feeding (Nyerhovwo, 2004)

Tapioca starch, an agricultural product, is a cheap substrate that easily available in tropical countries (Ramasamy *et al*., 2001).Tapioca starch can be isolated in a pure form with least contamination of non starchy constituents. Generally in industrial application, tapioca starch is used in paper, pharmaceutical and textiles industries as well as in manufacture of explosion adhesives and baby food (Kunamneni and Singh. 2005). The use of tapioca as a source of ethanol for fuel is already being exploited and found to be very promising (Nyerhovwo, 2004).

2.6.1 Starch Composition and Structure of Components

Amylose consists of anhydroglucose units that are linked by α-D-1, 4 glucoside bonds to form linear chains (Figure 2.3). Amylose molecules are typically made up from 200-2000 anhydroglucose units. The level of amylose and its molecular weight vary between different starches types. Aqueous solutions of amylose are unstable due to intermolecular attraction and association of neighboring amylose molecules. This leads to viscosity increase, retrogradation and precipitation of amylose particles (Hedley, 2002).

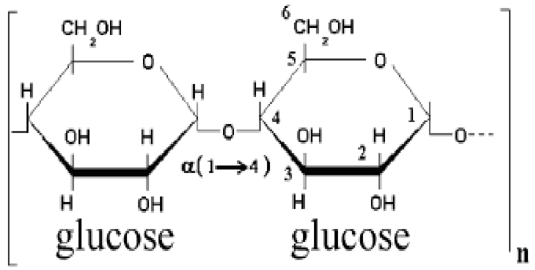

Figure 2.3: Chemical structure of Amylose. (Reis *et al*., 2002).

Amylopectin have a polymeric, branched structure (Figure 2.4).. Its consist of anhydroglucose units that are linked by α -D-1,6 bonds that occur every 20-30 anhydroglucose units. In addition, it also have α-D-1,4 bonds that are present in amylose.The level of amylopectin varies between different starches types. Aqueous solutions of amylopectin are characterized by high viscosity, clarity, stability and resistance to gelling (Hedley, 2002).

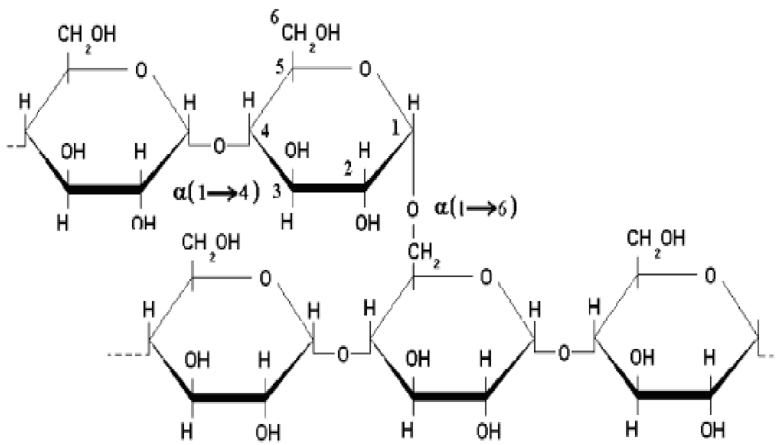

Figure 2.4: Chemical structure of amylopectin. (Reis *et al*., 2002).

2.7 Enzymatic Hydrolysis

There are two major processes of converting rich fermentable carbohydrates materials to ethanol which are enzymatic hydrolysis (from carbohydrates to sugars) and fermentation by microorganisms (from sugars to ethanol). The main role of enzymatic hydrolysis is to effectively provide the conversion of two major starch polymer components (amylose and amylopectin) to fermentable sugar that could subsequently converted to ethanol by yeast (Aggarwal *et al*., 2001). The degree of hydrolysis of native starch depends on the factors such as the substrate concentration, type and concentration of enzyme used, and on the applied process conditions such as pH, temperature and the mixing rate (Mojovic *et al*., 2006).

There are two processes in enzymatic hydrolysis; liquefaction and saccharification. The breakdown of large particles drastically reduces the viscosity of gelatinized starch solution, resulting in a process called *liquefaction*. The final stages of depolymerization are mainly the formation of mono-, di- , and tri-saccharides. This process is called *saccharification* (Barsby *et al*., 2003).

2.8 Fermentation

The generation of energy without the electron transport chain is called fermentation. This definition is the exact and original meaning of the term fermentation (Shuler and Kargi, 2002). Yeast convert glucose to ethanol and carbon dioxide fermentation, as shown by the following reaction:

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 \qquad \text{(Equation 2.1)}$$

Fermentation can be performed as a batch, fed batch or continuous process. The choice of most suitable process will depend upon the properties of microorganisms and type of starch (Chandel *et al*., 2007). There are many parameters should be concerned in fermentation process such as temperature, agitation speed, pH value, dissolved oxygen and nutrient. The effect of temperature and agitation speed on ethanol production is important for the successful progress of the fermentation.

2.8.1 Effect of Temperature

Temperature has a marked influence on the production of ethanol. According to Rivera *et al*., (2006) suitable temperature in fermentation process is the good condition for the yeast to react. Too high temperature kills yeast, and low temperature slows down yeast activity. Thus, to keep a specific range of temperature is required. Normally ethanol fermentation is conducted at temperature range between 30-35°C which stated by Shuler and Kargi, (2002) that the ethanol will be produced at highest concentration.

2.8.2 Effect of Agitation Speed

Agitation is important for adequate mixing, mass transfer and heat transfer. It assists mass transfer between the different phases present in the culture, also maintains homogeneous chemical and physical conditions in the culture by continuous mixing. Agitation creates shear forces, which affect microorganisms, causing morphological changes, variation in their growth and product formation and also damaging the cell structure (Kongkiattikajorn *et al*., 2007).

CHAPTER 3

MATERIALS & METHODOLOGY

3.1 Introduction

This chapter will discuss about the process for producing ethanol through fermentation process. In this study, bioethanol was produced while varying the effect of temperature and agitation speed during the fermentation processes. Meanwhile in enzymatic hydrolysis, liquefaction and saccharification processes are involved. The glucose consumption was observed by DNS method and the concentration of ethanol was determined using gas chromatography. The flowchart of the process is shown in Figure 3.1.

3.2 Framework of Ethanol Fermentation Process

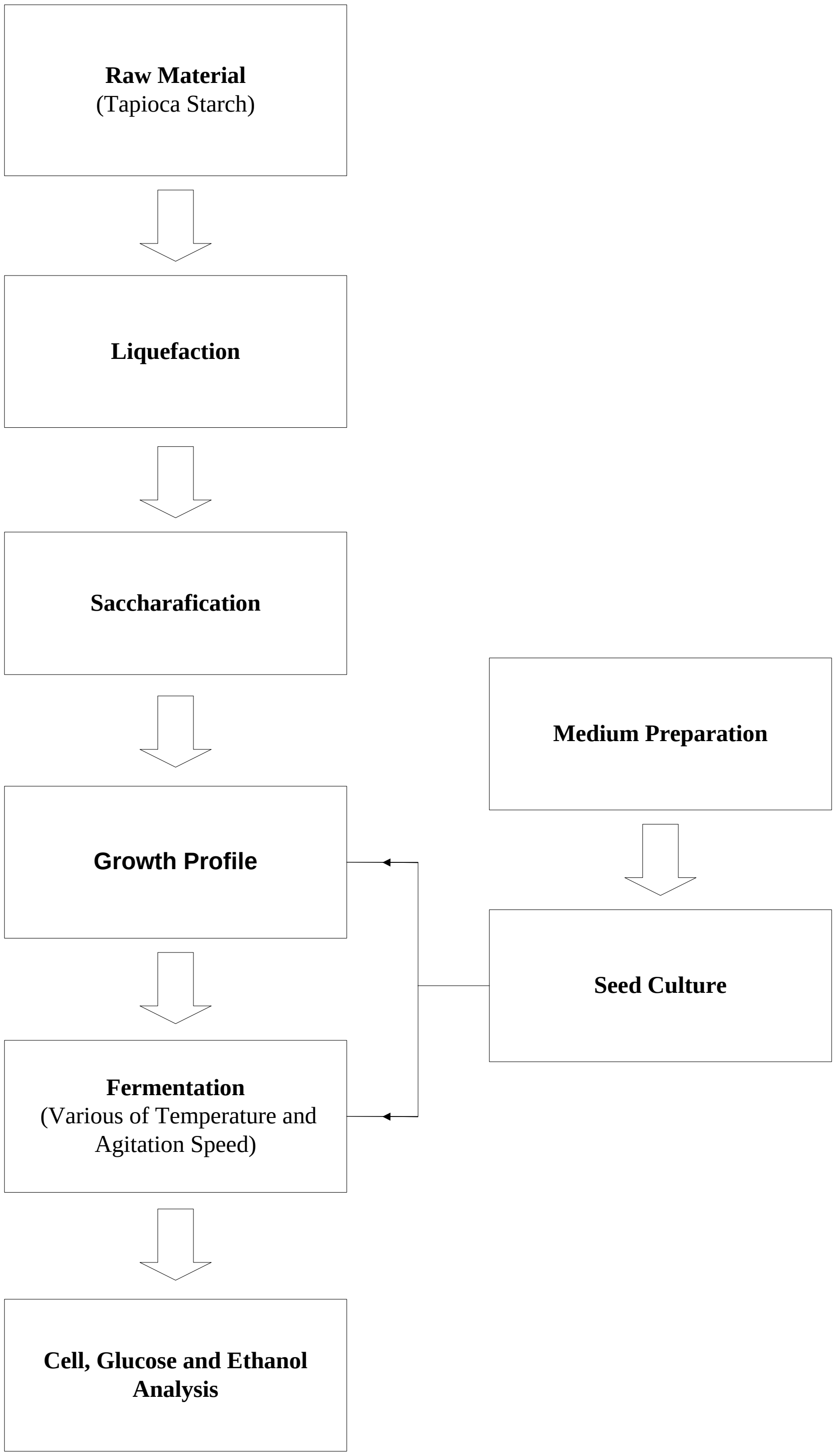

Figure 3.1: Framework of Ethanol Fermentation Process

3.3 Raw Materials

Starch from tapioca flour is utilized as starchy substance for the production of sugar.

3.4 Microorganisms

Saccharomyces cerevisiae is used as microorganism in bioethanol fermentation of tapioca starch.

3.5 Enzymatic Hydrolysis

3.5.1 Enzymes

α-Amylase from *Bacillus licheniformis* was used for liquefaction process. The enzyme activity was 144 KNU mL^{-1} (KNU, kilo novo units α-amylases— the amount of enzyme which breaks down 5.26 g of starch per hr according to Novozyme's standard method for the determination of α-amylase). Meanwhile, for the glucoamylase employed, its initial activity is 240 AGU/g (AGU is the amount of enzyme which hydrolyses 1 mmol of maltose per minute under specified conditions).

3.5.2 Hydrolysis Experiment

Tapioca starch, 100 g was mixed with water to get 33% w/v concentration. The mixture was hydrolyzed with enzymes (α-Amylase and glucoamylase) in two steps. Initially, the slurry of tapioca flour was liquefied with 80 μL of α-amylase at 85°C and pH 6.0 for 1 hr in water bath with 150 rpm agitation speed. Then,